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Award Number: W81XWH-10-1-0426

TITLE: Targeting Breast Cancer Recurrence via Hedgehog-Mediated Sensitization of Breast Cancer Stem Cells

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REPORT DATE: July 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*
01-07-2011

2. REPORT TYPE
Annual

3. DATES COVERED *(From - To)*
15 JUN 2010 - 14 JUN 2011

4. TITLE AND SUBTITLE
Targeting Breast Cancer Recurrence via Hedgehog-Mediated Sensitization of Breast Cancer Stem Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0426

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Dartmouth College
Hanover, NH 03755

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Abstract on next page.

15. SUBJECT TERMS
p63, stem cell, hedgehog, breast cancer, quiescence

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	24	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

14. ABSTRACT

Research supported by this award aims to determine if targeting the hedgehog signaling pathway in breast cancer can reduce breast cancer recurrence. In the United States and other countries with access to advanced cancer care, local and distant breast cancer recurrence accounts for ~95% of breast cancer mortality. Additionally, t he life-time risk of breast cancer recurrence is greater than the life-time risk of developing breast cancer in the general population. Together these two statements indicate that current treatments do not efficiently reduce the risk of disease recurrence. Disease recurrence is believed to be the result of a subset of tumor cells with two distinct features; broad-spectrum resistance to therapeutics and tumorigenicity. Our previous studies identified a regulatory relationship between ΔNp63α a protein that is required for long-term preservation of epithelial stem cells and the hedgehog-signaling pathway that governed stem cell quiescence. Stem cell quiescence is necessary to preserve long-term replicative capacity while simultaneously avoiding the detrimental effects of excessive proliferation. It is also a potent blockade to cellular differentiation. Based upon this we put forth the hypothesis that the hedgehog signaling pathway could be targeted to subvert quiescence in stem cell populations. Doing so would force these cells back into the cell cycle, possibly sensitizing them to adjuvant cancer therapeutics. To test this hypothesis we developed three specific tasks that are described in detail in the Statement of Work. Since this award was a collaborative IDEA award we sought to take advantage of the hedgehog-signaling expertise of Dr. David J. Robbins and the mammary stem cell and breast cancer expertise of Dr. James DiRenzo. What follows is a detailed description of progress towards the completion of the research supported by this award.

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Prologue: In support of Award number W81XWH-10-1-0430, we are pleased to present the following annual report detailing progress made towards the completion of work described in the Statement of Work associated with this award.

Introduction

The purpose of the research supported by this award is to determine if targeting the hedgehog signaling pathway in breast cancer can reduce breast cancer recurrence. Two specific facts about breast cancer recurrence highlight the need for better treatment modalities that target and prevent disease recurrence.

1. In the United States and other countries with access to advanced cancer care, local and distant breast cancer recurrence accounts for ~95% of breast cancer mortality (Jemal et al., 2010).
2. The life-time risk of breast cancer recurrence among survivors is greater than the life-time risk of developing breast cancer in the general population(Jemal et al., 2010).

Together these two statements suggest that while substantial progress has been made in treating primary breast cancer, those treatments do not efficiently reduce the risk of disease recurrence. Therefore there is a need for novel treatment strategies. Disease recurrence is believed to be the result of a subset of tumor cells with two distinct features; broad-spectrum resistance to therapeutics and tumorigenicity(Hurt and Farrar, 2008; Pardal et al., 2003; Polyak and Weinberg, 2009; Woodward et al., 2005). Our previous studies(Li et al., 2008) identified a regulatory relationship between ΔNp63α a protein that is required for long-term preservation of epithelial stem cells(Li et al., 2008; Mills et al., 1999; Yang et al., 1999) and the hedgehog-signaling pathway that governed stem cell quiescence. Stem cell quiescence is necessary to preserve long-term replicative capacity while simultaneously avoiding the detrimental effects of excessive proliferation. It is also a potent blockade to cellular differentiation(Coller et al., 2006). Based upon this we put forth the hypothesis that the hedgehog signaling pathway could be targeted to subvert quiescence in stem cell populations. Doing so would force these cells back into the cell cycle, possibly sensitizing them to adjuvant cancer therapeutics. To test this hypothesis we developed three specific tasks that are described in detail in the Statement of Work. Since this award was a collaborative IDEA award we sought to take advantage of the hedgehog-signaling expertise of Dr. David J. Robbins and the mammary stem cell and breast cancer expertise of Dr. James DiRenzo. What follows is a detailed description of progress towards the completion of the research supported by this award.

Research Accomplishments Associated with the Statement of Work

Task 1: Determine the effects of pharmacologic regulation of Smoothened on mammary stem cell quiescence.

Sub-Task 1: Evaluate the effects of Smo agonists on regulation of quiescence in Lin$^-$/CD24$^+$/CD29^{high} cells.

- A: Establish a treatment model based on one utilized according to the Frank-Kamenetsky model(Frank-Kamenetsky et al., 2002).
  - 1: Isolate and quantify Lin$^-$/CD24$^+$/CD29^{high} and Lin$^-$/CD24$^+$/CD29^{low} to determine if the ratio of mammary stem cells to committed mammary progenitors is altered by Smo activation.
  - 2: Evaluate mammosphere initiating capacity of Lin$^-$/CD24$^+$/CD29^{high} -/+ Smo agonist.

3: Determine the effects of Smo agonists on engraftment efficiency, ductal elongation and side-branching.

Progress: We have encountered multiple technical obstacles in our efforts to establish an efficient treatment model that was based on the Frank-Kamenetsky model. This model called for the administration of Smoothened agonists via oral gavage and while we were able to administer the drug via this method we did not observe increased expression of LacZ in the $Ptch1^{LacZ/+}$ mice following administration indicatong that regulation of the canonical hedgehog target gene, Patched1, was not altered in response to oral-gavage of Smoothened agonists. In vitro testing of the compounds indicated that they were able to activate endogenous expression of Patched1, suggesting that they are able to activated hedgehog signaling. The most likely reason for the inability to detect hedgehog activation following administration of the smoothened agonists via oral gavage is that the compounds may have poor pharmacokinetic or pharmacodynamics properties on the C57Bg/129 genetic background. Our solution to this problem was to adopt a genetic strategy to determine if hyperactivation of hedgehog signaling via loss of Ptch1 would:

1. Alter ratios of mammary stem cells to mammary progenitors.
2. Alter mammosphere-initiating capacity.
3. Alter engraftment efficiency or ductal elongation

Our rationale for this was that genetic activation of hedgehog signaling via Patched1 heterozygosity would mimic the effects of pharmacologic activation or smoothened. Therefore we would still be able to address whether or not hedgehog activation had any effect on mammary stem cell activity. We isolated mammary epithelial cells (MECs) from wt and Ptch+/- mice by generating a single cell suspension from freshly dissected mammary glands. Quantification of enriched fractions of mammary stem cells ($Lin^-/CD24^+/CD29^{high}$) and mammary progenitors ($Lin^-/CD24^+/CD29^{low}$) indicated an increase in the luminal progenitor population, and a decrease in the mammary stem cell population in the Ptch1+/- when relative to wt mice (Figure 1). These results are consistent with the previous finding that mammary stem cells from $Ptch1^{LacZ/+}$ mice are defective in quiescence and they support a model in which hedgehog signaling promotes the activation of the mammary regenerative hierarchy.

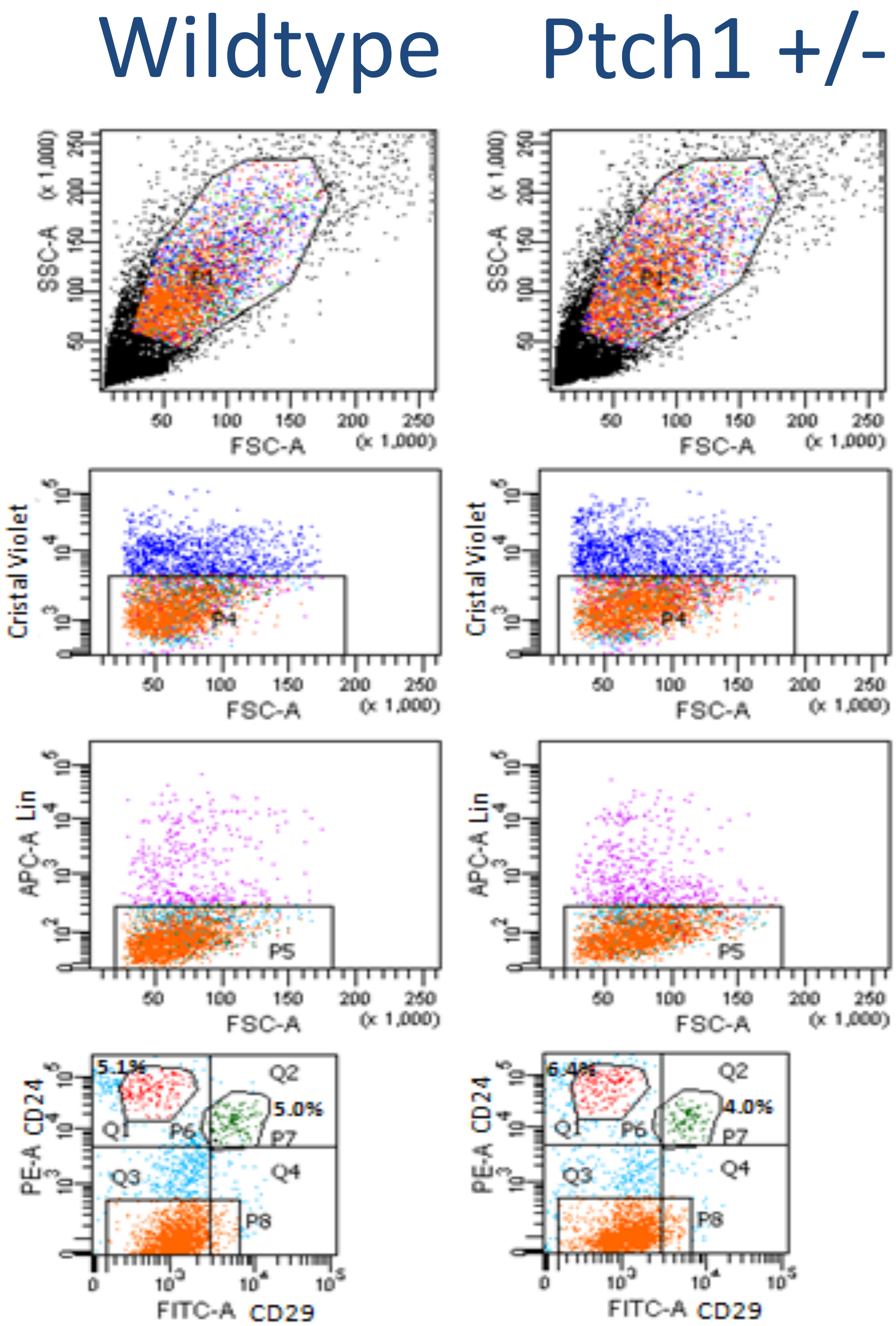

Figure 1: Quantitative comparison of enriched fractions of mammary stem cells ($Lin^-/CD24^+/CD29^{high}$) and luminal epithelial progengitors ($Lin^-/CD24^+/CD29^{low}$) indicates increased elaboration of committed progenitors by committed stem cells. Data above indicate the prospective sorting strategy that was used to isolate $Lin^-/CD24^+/CD29^{high}$ and $Lin^-/CD24^+/CD29^{low}$ fractions. Results indicate a 20% decline in the size of the mammary stem cell pool and a 25% increase in the mammary progenitor pool in $Ptch1^{LacZ/+}$ mice compared to wild-type littermates. This result is consistent with the hypothesis that hedgehog signaling activation results in increased mammary stem cell activity.

Consistent with the observation that hedgehog activation lead to increased mammary stem cell activity we also observed that enriched fractions of mammary stem cells from Ptch1$^{LacZ/+}$ formed mammospheres with increased efficiency relative to the same fraction from wild-type littermates. (Figure 2).

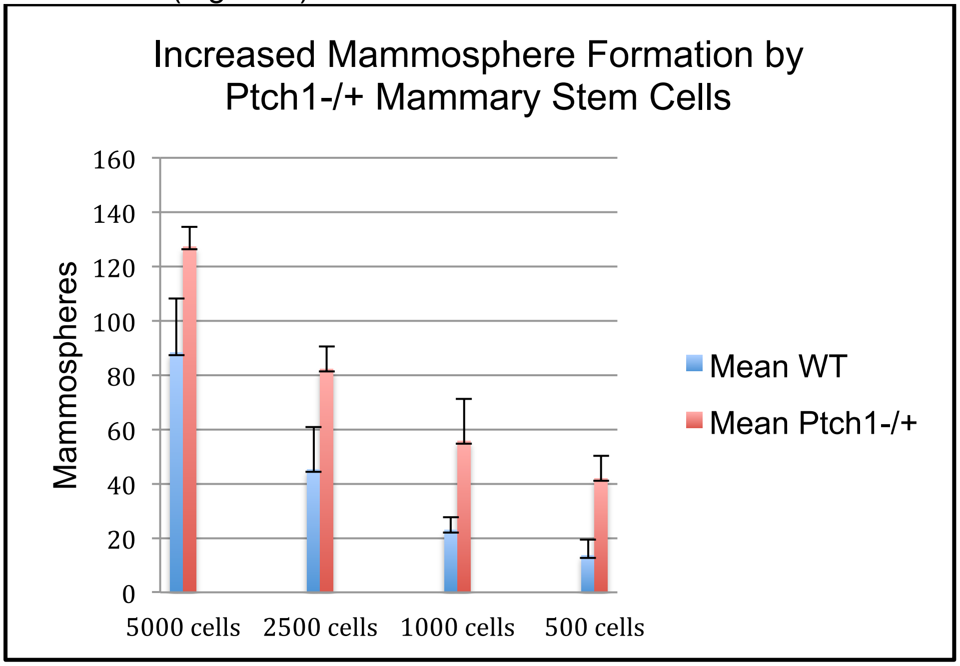

Figure 2: Comparative analysis of mammosphere forming capacity of enriched fractions of mammary stem cells from Ptch1$^{LacZ/+}$ and wild-type littermates. Lin$^-$/CD24$^+$/CD29^{high} cells were isolated from 3 Ptch1$^{LacZ/+}$ and 3 wt littermates and cultured in ultra-low attachment culture dishes for 12 days. Spheres were stained with the vial fluorescent dye, Calcien EM and counted. Data represent the mean of three distinct experiments and error bars represent the standard deviation. Results indicate that hedgehog activation via Patched1 heterozygosity leads to increased mammosphere forming activity.

To better understand the biological significance of the increased mammary stem cell activity in the Ptch1$^{LacZ/+}$ mouse relative to age-matched wild-type mice we transplanted enriched fractions of mammary stem cells (Lin$^-$/CD24$^+$/CD29^{high}) from Rosa26 and Ptch1$^{LacZ/+}$ mice into the cleared fat pads of syngeneic hosts. Rosa26 mice were used in place of wild-type C57/B6 mice because the constituative expression of Lacz would enable unambiguous detection of donor-derived mammary outgrowths. Results indicated that Lin$^-$/CD24$^+$/CD29^{high} cells from Ptch1$^{LacZ/+}$ mice gave rise to ductal outgrowths with significantly more side-branching and precocious lobulo-alveolar formation (Figure 3)

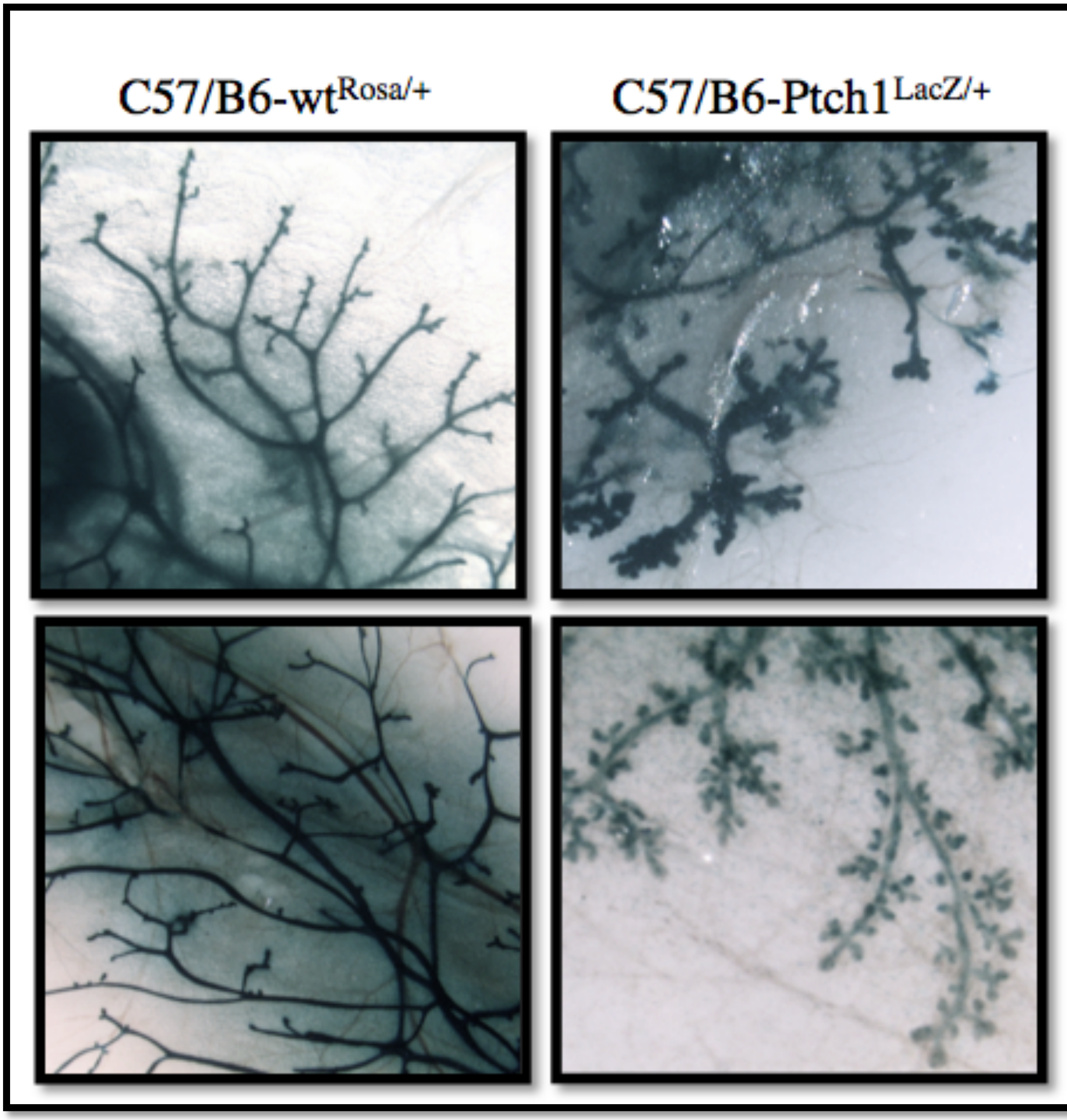

Figure 3: Enhanced side-branching and lobulo-alveolar formation in ductal outgrowths derived from Ptch1$^{LacZ/+}$ mammary stem cells. One hundred Lin^{-}/CD24^{+}/CD29^{high} cells from either Ptch1$^{LacZ/+}$ or Rosa26 mice were transplanted into the cleared fat pads of syngeneic recipients. At 6 weeks post transplant mammary wholemounts were prepared and stained with X-gal to detect donor-derived ductal outgrowths. Results indicate that constituative activation of hedgehog signaling lead to activation of the mammary regenerative hierarchy.

Collectively these data support the conclusion that hedgehog activation stimulates mammary stem cell activity, thereby driving the mammary regenerative hierarchy. Additionally the premature lubolo-alveolar formation is consistent with a model in which hedgehog-mediated activation of mammary stem cells results in elaboration of a luminal epithelial lineage. To test this enriched fractions of mammary stem cells (Lin^{-}/CD24^{+}/CD29^{high}) from wild-type and Ptch1$^{LacZ/+}$ littermates were cultured under conditions that promote the expansion of luminal epithelial colonies, myoepithelial colonies and mixed lineage colonies and colonies were stained with CK8 and CK5 to distinguish luminal epithelia from myoepithelia. This enabled to quantification of developmental endpoints and revealed that mammary stem cells from the Ptch1$^{LacZ/+}$ mouse model strongly favored the luminal epithelial cell fate over the mixed lineage cell fate (Figure 4).

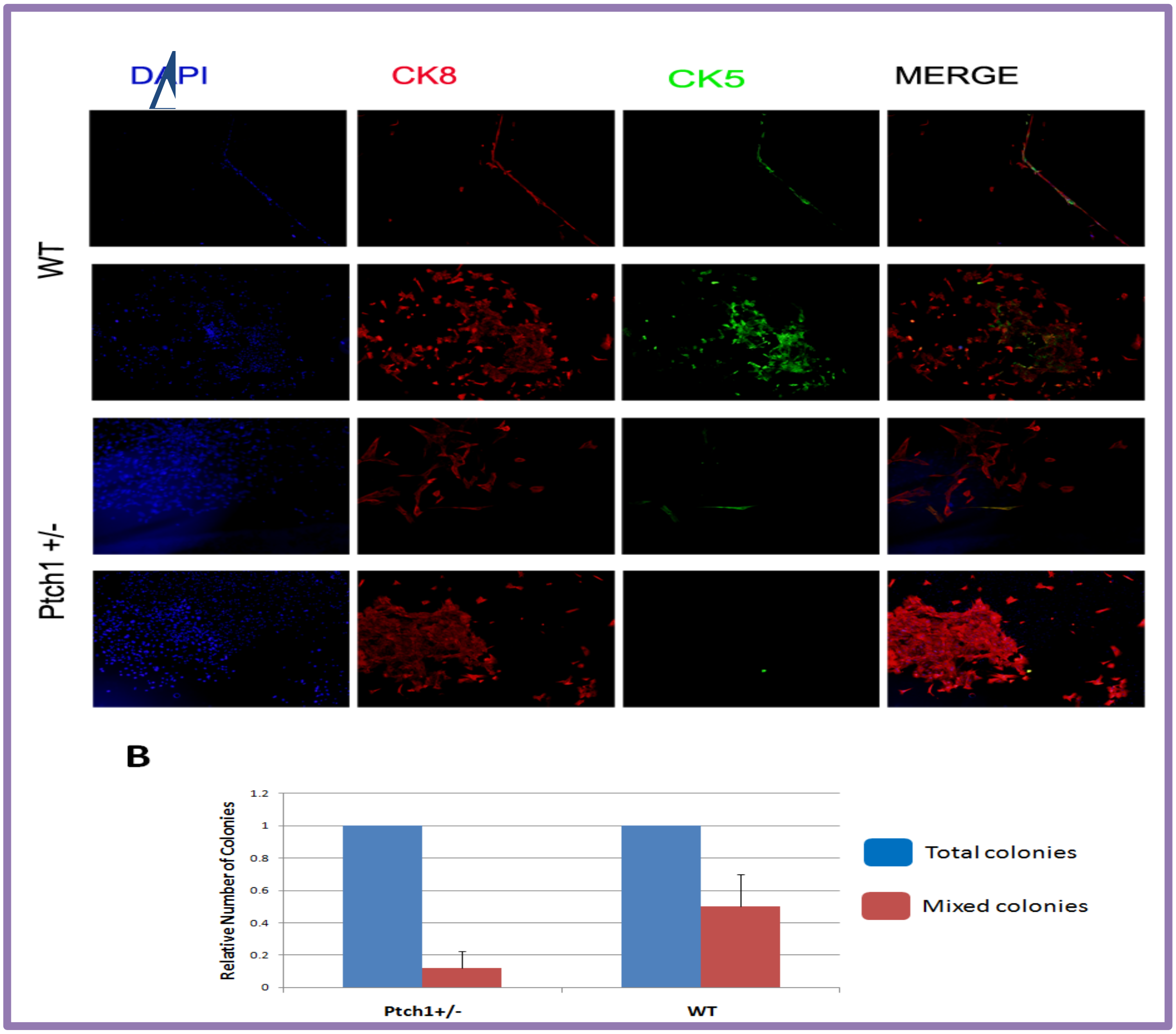

Figure 2. Aberrant activation of Hedgehog pathway result in the transition from mammary stem cell to luminal progenitor cell. A. Two representative colonies for each mice group (wt and Ptch1+/-) stained for CK5 and CK8, myoepithelial and luminal markers respectively. An absence of myoepithelial cells within the colony was observed in the Ptch1+/- colonies when compare to the wt. **B.** The relative number of mixed colonies in red (in which both luminal and myoepithelial cells are present), compare to the total number of colonies observed (Error bars represent standard deviation in technical replicates). The decrease in the number of myoepithelial cells in the heterozygous mice is indicative of the commitment of the stem cell population to luminal progenitors when HH pathway is activated.

Sub-Task 2: Evaluate the ability of Smo antagonists to rescue the Ptch1$^{-/+}$ phenotype.

A: Identical treatment model.

1: Isolate and quantify Lin$^-$/CD24$^+$/CD29^{high} and Lin$^-$/CD24$^+$/CD29^{low} to determine if the ratio of mammary stem cells to committed mammary progenitors is altered by Smo antagonism.

2: Evaluate mammosphere initiating capacity of Lin$^-$/CD24$^+$/CD29^{high} from Ptch1$^{-/+}$ mice -/+ Smo antagonist.

3: Determine the effects of Smo agonists on engraftment efficiency, ductal elongation and sidebranching.

Progress: Similar to the studies involving in vivo administration of Smoothened Agonists we have encountered pharmacokinetic obstacles that have limited the biological activity of the Shoothened Antagonist, SANT1. Our in vitro studies indicated that the drug does oppose hedheghog signaling however we have seen no evidence of changes in hedgehog signaling status in vivo folloing administration of SANT1. To circumvent this problem and to address the effects of Smoothened inhibition on hedgehog signaling in mammary stem cells, we have purchased 5 lentiviral shRNA constructs designed to produce a knock-down of Smoothened. At the time of this report those constructs are being packaged into lentiviral particles and tested in vitro to determine which produced the most significant suppression of Smoothened expression. Once this is complete we intend to infect Lin$^-$/CD24$^+$/CD29^{high} cells from wild-type and Ptch1$^{LacZ/+}$ mice with the selected viral particle and transplant those cells into cleared fat pads of syngeneic recipients. Our prediction is that suppression of Smoothened will rescue the aberrant side-branching and lobulo-alveolar formation phenotype in the Ptch1$^{LacZ/+}$ mouse. Additionally we predict that suppression of Smoothened in wild-type Lin$^-$/CD24$^+$/CD29^{high} cells will lead to diminished ductal elongation and side-branching.

Progress on this sub-task has been remarkably challenging due to the limitation of the smoothened antagonists and the labor-intensive process of identifying the optimal lentiviral shRNA for suppression of Smoothened.

Task 2: <u>Determine if hedgehog-mediated defective quiescence predisposes mulliparous MMTV-myc mice to tumorigenesis.</u>

Sub-Task 1: Genetic analysis: Cross FV/b MMTV-myc with B6/129 Ptch1$^{-/+}$

A: 25% Ptch1$^{+/+}$ + No MMTV-myc Tg (WT on Mixed FV/bxB6/129 bkg)

B: 25% Ptch1$^{-/+}$ + No MMTV-myc Tg (Ptch on Mixed FV/bxB6/129 bkg)

C: 25% Ptch1$^{+/+}$ + MMTV-myc Tg (MMTV-myc on Mixed FV/bxB6/129 bkg)

D: 25% Ptch1$^{-/+}$ + MMTV-myc Tg (Ptch1$^{-/+}$ + MMTV-myc Mixed FV/bxB6/129 bkg)

1: Collect 8 females from each genotype and monitor for tumorigenicity over 12 months.

Progress: We have completed the genetic crosses necessary to produce a minimum of 8 mice wth each genotype. We have also developed a Q-PCR-based assay that can identify Ptch1$^{LacZ/+}$ from wild-type and MMTV-myc Tg from wild-type. These mice are evaluated twice weekly by the PI and daily by the staff at the Animal Resource Facility. Presently we have observed tumors in 4 of the mice that are Ptch1$^{LacZ/+}$ and MMTV-myc Tg and 1 in mice that are Ptch1$^{+/+}$ and MMTV-myc Tg. Monitoring will continue through June of 2012 to ensure that the youngest these mice reach 1 year of age. These preliminary results may suggest that activation of hedgehog signaling via Ptch1 heterozygosity is promoting c-myc-mediated tumorigenesis in nulliparous mice, however at the present we can not make that assertion conclusively. Table 1 (below) summarizes the data from these genetic studies.

Genotype	Number	Confirmed Tumorigenesis	Average age of Onset
Ptch1$^{+/+}$ + MMTV-myc$^{-/-}$	11	0	N/A
Ptch1$^{+/+}$ + MMTV-myc$^{+/-}$	10	1	7 months 2 days
Ptch1$^{LacZ/+}$ + MMTV-myc$^{-/-}$	8	0	
Ptch1$^{LacZ/+}$ + MMTV-myc$^{+/-}$	13	4	6 months 13 days

All tumors have been dissected fixed and embedded in paraffin for histologic analysis.

Sub-Task 2: "Pseudo-genetic analysis"

- A: Isolate Lin^{-}/CD24^{+}/CD29^{high} from MMTV-myc and infect with pGIPZ-non-specific shRNA or pGIPZ-mPtch1 shRNA
- B: Sort GFP-ve from GFP+ve.
- C: Transplant GFP+ve cells into cleared fat pads of 3 week old FV/B recipients
  - 1: Transplant 16 glands (i.e. 8 recipient mice) with Lin^{-}/CD24^{+}/CD29^{high} infected with pGIPZ-non-specific shRNA.
    - a: Monitor transplants for tumorigenesis over 12 months.
  - 2: Transplant 16 glands from 8 mice with Lin^{-}/CD24^{+}/CD29^{high} infected with with pGIPZ-mPtch1 shRNA.
    - a: Monitor transplants for tumorigenesis over 12 months.

Progress: Efforts to target expression of Patched1 in mammary stem cells from wild-type and MMTV-myc mice with lentiviral shRNA have been hampered by poor infection efficiency. Despite significant efforts to improve viral titers and optimize the physical and chemical conditions necessary for high efficiency we have consistently achieved less than 10% infection efficiency. Under these conditions it has not been possible to detect any suppression of Patched1. These poor efficiencies have made it difficult to isolate sufficient numbers of GFP-positive cells to continue with transplantation. Since the goal of the sub-task is to determine if there is a genetic interaction between hedgehog signaling and c-myc-mediated breast tumorigenesis, we are confident that the progress described above in Sub-task 1 will address that question.

Task 3: Evaluate the ability of hedgehog activation to sensitize tumor stem cells to taxanes in vitro and in vivo.

Sub-Task 1: In vitro studies

- A: Isolate Lin^{-}/CD24^{+}/CD29^{high} from wt and Ptch1$^{-/+}$ mice and evaluate the effects of paclitaxel on mammosphere formation.
- B: Isolate Lin^{-}/CD24^{+}/CD29^{high} from wt mice and evaluate the ability of a Smoothened agonists to sensitize cells to paclitaxel.
  - 1: Isolation of Lin^{-}/CD24^{+}/CD29^{high} from wt mice
  - 2: Pre-treatment with paclitaxel.
  - 3: Culture under low-binding conditions that favor expansion of self-renewing populations.
- C: Isolate Lin^{-}/CD24^{+}/CD29^{high} from Ptch1$^{-/+}$ mice and evaluate the ability of Smo antagonist to protect cells from paclitaxel.

Sub-Task 2: In vivo studies

A: Determine the efficiency of serial transplantation of tumors derived from MMTV-myc and Ptch1$^{-/+}$ x MMTV-myc following paclitaxel treatment.

1: Cross FV/b MMTV-myc with B6/129 Ptch1$^{-/+}$

a: 25% Ptch1$^{+/+}$ + No MMTV-myc Tg

b: 25% Ptch1$^{-/+}$ + No MMTV-myc Tg

c: 25% Ptch1$^{+/+}$ + MMTV-myc Tg

<u>Progress:</u> Efforts to determine whether hedgehog activation via the use of Smoothened agonists is sufficient to sensitize mammary stem cells to the spindle poison, paclitaxel are under way and several observations have been made.

First we noted that while mammary stem cells are resistant to paclitaxel in vivo, they are not when cultured in vitro. This difference very likely reflects the fact that cells from the Lin^{-}/CD24^{+}/CD29^{high} fraction are able to proliferate in culture which is inconsistent with their ability to retain BrdU for prolonged periods of time in vivo. This difference is also likely to reflect the protective effects of the mammary stem cell niche and the loss of these effects when cells are removed from the body of the mice. To address this we attempted to establish a sub-threshold dose of paclitaxel that was sufficient to cause the depolymerization of the mitotic tubule but not kill the cells in vitro. Our data indicate that while the sub-threshold doses of paclitaxel would support prolonged survival of mammary stem cells in culture, any dose that was sufficient to disrupt mitotic spindle activity was also sufficient to kill the Lin^{-}/CD24^{+}/CD29^{high} cells. These observations are consistent with our over-arching hypothesis that it is the state of proliferative quiescence observed within mammary stem cells in vivo that confers protection from cytotoxic chemotherapeutics. These studies strongly suggest that the only way to determine if hedgehog activators are able to sensitize mammary stem cells will be to determine if in vivo administration of these compounds is sufficient to subvert long-term label retention, as was noted in the Ptch1$^{LacZ/+}$ mouse. This study is a derivative of that which is described in Sub-task 2A above in which we will take advantage of the known defect in cellular quiescence in mammary stem cells of Ptch1$^{LacZ/+}$ mice. Our goal here will be to ask of this quiescence defect sensitizes Lin^{-}/CD24^{+}/CD29^{high} cells to paclitaxel which will result in a decrease in engraftment efficiency and ductal outgrowth. Our goal is to begin paclitaxel treatment by 4/1/2012 and complete the experiment by early June of 2012.

<u>Key Research Accomplishments</u>

1. Demonstration of a shift in the ratio of mammary stem cells to mammary progenitors in wild-type vs Ptch1$^{LacZ/+}$ mice.

2. Demonstration of dramatically enhanced luminal epithelial commitment in response to constituative hedgehog signaling in the Ptch1-/+ mouse.

3. Demonstration of enhanced formation of luminal mammospheres at the expense of bi-potent spheres by mammary stem cells from Ptch1-/+ mice relative to those from wild-type littermates.

4. Demonstration of a proliferative advantage, or quiescence defect in mammary stem cells in which hedgehog signaling is persistent.

5. Demonstration that the aberrant side-branching and precocious lobulo-alveolar development observed in the Ptch1$^{LacZ/+}$ mouse is a mammary stem cell autonomous effect.

6. Development of cohorts of MMTV-myc$^{+/-}$/Ptch1$^{+/+}$ and MMTV-myc$^{+/-}$/Ptch1$^{+/LacZ}$ for the determination of whether defective mammary stem cell quiescence is condition of breast cancer predisposition.

7. In vitro expansion of enriched fractions of mammary stem cells (Lin^{-}/CD24^{+}/CD29^{high}) by culturing on irradiated NIH3T3 cells.

8. Achieved ectopic expression of c-myc in mammary stem cells from wild-type and Ptch1-/+ mice via retroviral transduction.

Reportable Outcomes

1. Publication of an article by Kent et al (attached as an appendix) in Cell Cycle describing the influence of Notch signaling on mammary stem cell quiescence. This research was a direct product of the findings associated with this award. More specifically our characterization of the molecular basis underlying the quiescence defect observed in the Ptch1$^{+/LacZ}$ lead to the identification of Notch3 as a transcriptional target of ΔNp63α and a mediator of cellular quiescence in mammary stem cells.

2. Presentation of a poster describing progress associated with this award at the 2011 Era of Hope Conference in Orlando Florida

3. Seminar presentation at the University of Massachusetts describing the genetic interactions between ΔNp63α, hedgehog signaling and notch signaling. Much of the work described in this seminar was the product of research supported by this award.

4. Seminar presentation at the University of Vermont describing the genetic interactions between ΔNp63α, hedgehog signaling and notch signaling. Much of the work described in this seminar was the product of research supported by this award.

5. Seminar presentation at the Jackson Laboratory describing the genetic interactions between ΔNp63α, hedgehog signaling and notch signaling. Much of the work described in this seminar was the product of research supported by this award.

Conclusions

1. Data derived from this study support a model in which activation of hedgehog signaling is sufficient to stimulate mammary regenerative stasis. This conclusion is supported by the following findings:
   a. Activation of hedgehog signaling via Ptch1 heterozygosity lead to:
      i. Increased elaboration of mammary progenitors relative to mammary stem cells.
      ii. Increased specification of luminal epithelial differentiation.

iii. Increased mammosphere forming capacity

b. Transplantation of mammary stem cells derived from wild-type vs Ptch1 heterozygous mice resulted in increased side-branching and precocious lobuloalveolar development.

2. Preliminary data derived from this study indicate that activation of hedgehog signaling is sufficient to confer tumorigenesis upon nulliparous MMTV-myc mice. Kindreds associated with this study have not reached 1 year of age and so at this point no firm conclusions can be drawn.
3. Loss of quiescence in mammary stem cells confers sensitivity to paclitaxel. This conclusion is being tested more rigorously in ongoing studies.

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Cell Cycle 10:18, 3111-3118; September 15, 2011; © 2011 Landes Bioscience

ΔNp63α promotes cellular quiescence via induction and activation of Notch3

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Key words: p63, Notch, quiescence, stem cell

Genetic analysis of TP63 indicates that ΔNp63 isoforms are required for preservation of self-renewing capacity in the stem cell compartments of diverse epithelial structures; however, the underlying cellular and molecular mechanisms remain incompletely defined. Cellular quiescence is a common feature of adult stem cells that may account for their ability to retain long-term replicative capacity while simultaneously limiting cellular division. Similarly, quiescence within tumor stem cell populations may represent a mechanism by which these populations evade cytotoxic therapy and initiate tumor recurrence. Here, we present evidence that ΔNp63α, the predominant TP63 isoform in the regenerative compartment of diverse epithelial structuresm, promotes cellular quiescence via activation of Notch signaling. In HC11 cells, ectopic ΔNp63α mediates a proliferative arrest in the 2N state coincident with reduced RNA synthesis characteristic of cellular quiescence. Additionally, ΔNp63α and other quiescence-inducing stimuli enhanced expression of Notch3 in HC11s and breast cancer cell lines, and ectopic expression of the Notch3 intracellular domain ($N3^{ICD}$) was sufficient to cause accumulation in G_0/G_1 and increased expression of two genes associated with quiescence, Hes1 and Mxi1. Pharmacologic inhibition of Notch signaling or shRNA-mediated suppression of Notch3 were sufficient to bypass quiescence induced by ΔNp63α and other quiescence-inducing stimuli. These studies identify a novel mechanism by which ΔNp63α preserves long-term replicative capacity by promoting cellular quiescence and identify the Notch signaling pathway as a mediator of multiple quiescence-inducing stimuli, including ΔNp63α expression.

Introduction

Cellular quiescence is implicated in maintenance of adult stem cells, and evidence indicates that defective quiescence leads to exhaustion of the stem cell pool.[1-7] Prolonged tissue stasis is achieved by coordinated regulation of regenerative hierarchies initiated by asymmetric division of an adult stem cell to produce mitotic offspring fated to retain or forfeit self-renewing capacity. While adult stem cells retain proliferative capacity, accumulating evidence indicates that they utilize cellular quiescence to restrict the number of divisions they undergo and to resist differentiation.[8-10] Pulse labeling with nucleotide analogs has identified long-term label-retaining cells that have subsequently been shown to co-enrich with adult stem cells.[4,11-16] Similarly, inducible expression of a GFP-histone2B fusion protein has enabled isolation of cells based on label retention and the subsequent demonstration that these cells possess potent stem cell activity.[17-19] Slow-cycling or non-cycling cells within tumor populations selectively exhibit chemo-resistance and tumor-initiating capacity, suggesting that quiescence is a common feature among tumor stem cell populations[20-23] and implying that pharmacologic disruption of stem cell quiescence in the setting of adjuvant therapeutics might reduce rates of cancer recurrence. Quiescence is an active process involving overlapping programs of gene regulation in response to distinct quiescence-inducing stimuli.[11] Studies using fibroblasts identified serum deprivation early response genes (SDERGs) that were not coordinately repressed by serum stimulation, indicating a unique transcriptional response to serum deprivation.[24] Among the SDERGs, Notch3 was activated in less than one hour following serum deprivation. The canonical Notch target gene, Hes1 is activated in response to quiescence and is sufficient to maintain the reversible nature of quiescence.[25] These studies coupled to functional analysis of Notch signaling in MaSCs[26] implicate Notch signaling in adult stem cell quiescence.

The Notch signaling pathway executes context-dependent cell-fate decisions in diverse tissue types during embryonic patterning, stem cell regulation, proliferation, differentiation and apoptosis.[27-31] Notch activity is implicated in numerous malignancies and has been shown to be either an oncogene[32,33] or a tumor suppressor[28,34] in distinct cell types. It also governs aspects of development and tumorigenesis in the mammary gland.[27] Disruption of Notch signaling in the mammary gland via conditional deletion of RBPj (CBF1), causes expansion of the basal/myoepithelial cells and a concomitant loss of luminal epithelial cells, indicating that Notch signaling was important for luminal cell fate determination.[35] Additionally, suppression of CBF1 in enriched fractions of mammary stem cells (MaSCs) resulted in increased proliferation and mammary regenerative activity.[26]

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Submitted: 07/11/11; Accepted: 07/11/11
DOI: 10.4161/cc.10.18.17300

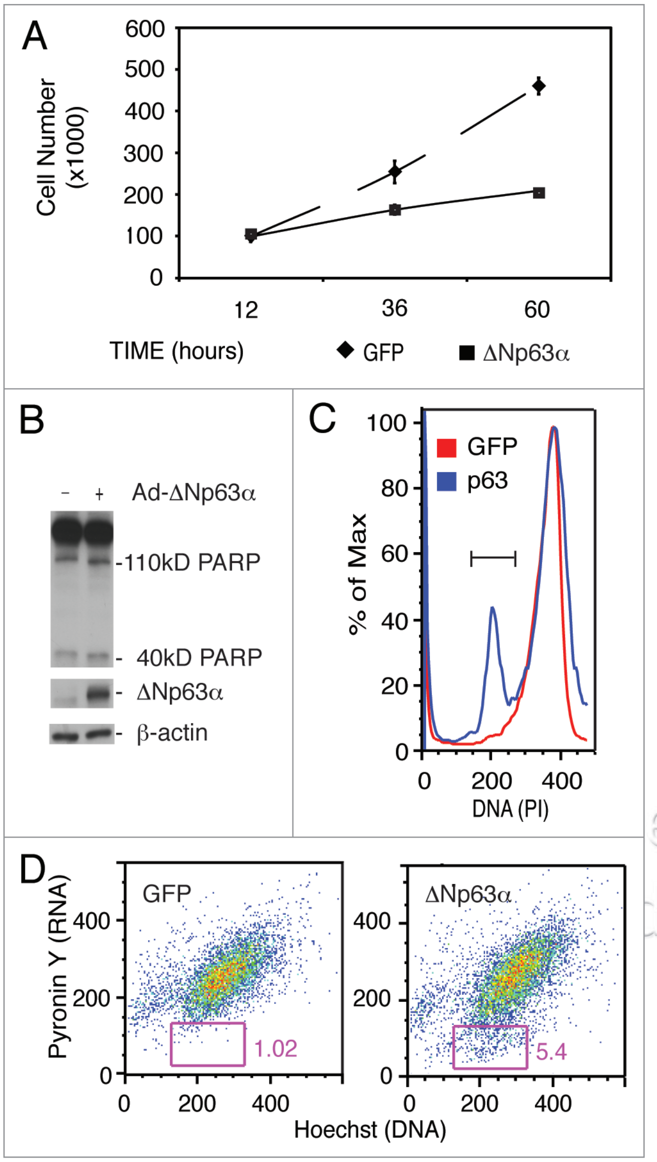

Figure 1. ΔNp63α promotes cellular quiescence in HC11 cells. (A) ΔNp63α suppresses proliferation of HC11 cells. Cells were infected with Adenovirus expressing ΔNp63α or GFP and counted daily beginning 12 h after plating and infection. (Error bars represent standard deviation from two experiments). (B) Suppression of proliferation by ΔNp63α is not a result of apoptosis. Following 48 h of adenoviral infection of ΔNp63α or GFP, protein lysates were derived from all cells in the culture, and apoptosis was measured by protein gel blotting for PARP and cleaved PARP products indicative of apoptotic events. β-actin served as a loading control. (C) ΔNp63α arrests HC11 cells in a state of 2N DNA content. Following 24 h of adenoviral expression of ΔNp63α or GFP and 24-h thymidine synchronization, cells were washed and fed in the presence of nocodazole. A subpopulation of cells overexpressing ΔNp63α arrested in G_0/G_1 of the cell cycle as evidenced by propidium iodide staining. (D) Cell cycle arrest induced by ΔNp63α corresponds to the induction of cellular quiescence. Following ΔNp63α or GFP overexpression, cells were fixed and assessed for a reduction in RNA content, indicative of cellular quiescence. Representative experiments are shown for (C and D).

Together, these results indicate that Notch signaling in the mammary gland contributes to at least two distinct cell fate decisions, luminal vs. basal/myoepithelial differentiation and quiescence vs. activation in MaSCs. Constituative Notch signaling in the mammary gland results in a developmental blockade associated with decreased elaboration of alveoli during pregnancy, and this phenotype is believed to underlie tumorigenesis in this model.[36] Other studies have associated Notch signaling with the oncogenic activity of the Wnt signaling pathway in the mammary gland.[37]

Genetic analysis clearly indicates that ΔNp63 products of *TP63* are required for preservation of self-renewal in diverse epithelial structures.[38,39] More recent studies have indicated that ΔNp63α is a potent blockade to cellular senescence;[40] however, it is unclear if this activity accounts for retention of proliferative capacity in adult stem cells. Here, we provide evidence that ΔNp63α promotes cellular quiescence via the induction of Notch3 expression and activity. ΔNp63α is sufficient to promote cellular quiescence, and Notch3 expression is induced by ectopic ΔNp63α and other quiescence-inducing stimuli in HC11 cells. Other data indicate that ectopic activation of Notch signaling is sufficient to restrict proliferation in a manner that is independent of ectopic ΔNp63α, and that genetic and pharmacologic repression of Notch signaling is sufficient to subvert cellular quiescence induced by ectopic ΔNp63α or other quiescence-inducing stimuli. Our findings identify Notch signaling as a mediator of cellular quiescence and provide a novel mechanism by which ΔNp63α mediates cellular quiescence and preservation of replicative capacity in adult stem cells.

Results

ΔNp63α promotes cellular quiescence in HC11 cells. Previously, we reported a genetic interaction between ΔNp63α and hedgehog signaling, demonstrating that ΔNp63α preserves long-term replicative capacity via promotion of cellular quiescence.[10] To further test this conclusion and characterize the mechanism(s) underlying ΔNp63α-mediated quiescence, we adopted the HC11 cell line, which is an immortalized model of MaSCs possessing mammary regenerative capacity. Ectopic ΔNp63α caused a significant reduction in cell number (**Fig. 1A**) with no observable increase in PARP cleavage (**Fig. 1B**), indicating that ectopic ΔNp63α results in proliferative arrest and not apoptosis. To determine if the proliferative arrest was cellular quiescence, we monitored cell cycle progression of HC11s with ectopic GFP or ΔNp63α. An 18-h thymidine blockade resolved populations that had or had not traversed the G_1/S checkpoint. Following this blockade, cells were released and allowed to progress to a nocodazole block in G_2. Cell cycle distribution analysis indicated that ectopic ΔNp63α, but not GFP, produced a fraction of cells that failed to progress to and traverse the G_1/S boundary (**Fig. 1C**). This result indicates that ΔNp63α was sufficient to arrest cells in the 2N state under conditions in which there was sufficient mitogenic stimulation for GFP-expressing cells to progress to S phase. A common feature of cellular quiescence is decreased RNA biosynthesis, which can be detected by staining cells with pyronin Y.[41] To determine whether the ΔNp63α-mediated accumulation in G_0/G_1 was the result of increased cellular quiescence, cells expressing ΔNp63α or GFP were stained with the DNA dye Hoechst-33342 and pyronin Y and analyzed by flow cytometry. Results (**Fig. 1D**) indicate that ectopic ΔNp63α resulted in accumulation of a pyronin Y^{low} subset of 2N cells. These results demonstrate that ΔNp63α is able to promote cellular quiescence, suggesting a cellular mechanism by which it preserves long-term replicative capacity.

Notch3 is induced by ectopic ΔNp63α and other quiescence-inducing stimuli. The previous result, coupled to studies linking Notch to TP63[42,43] and to quiescence in fibroblasts[24] and MaSCs,[26] suggested that Notch signaling may contribute to ΔNp63α-mediated quiescence. To test this, we sought to determine the effects of ectopic ΔNp63α on expression of all four Notch family members. Results indicated that in HC11 cells, Notch3 expression increased in response to ectopic ΔNp63α, while Notch1 and Notch2 were unaffected, and Notch4 expression declined (**Fig. 2A**). Induction of Notch3 by ectopic ΔNp63α was also observed in MCF7 cells (**Fig. 2B**), suggesting that regulation of Notch3 by ΔNp63α may be a common event in mammary epithelium. These results indicated a regulatory relationship between ΔNp63α and Notch3, which further suggested that ΔNp63α may contribute to Notch signaling. Protein gel blot analysis of Notch3 in HC11 cells overexpressing either GFP or ΔNp63α indicated that ectopic ΔNp63α caused increases in both full-length Notch3 and the truncated intracellular domain (**Fig. 2C**). Similarly, ectopic ΔNp63α was sufficient to increase expression of Hes1 (**Fig. 2D**). Together, these results indicate that ΔNp63α increases expression of Notch3 and activation of the Notch signaling pathway. These results coupled to studies linking ΔNp63α to quiescence[10] and Notch to quiescence[11,24,43] suggest that Notch3 expression is a common feature of cellular quiescence. To test this, HC11s cells were cultured under normal monolayer conditions or under three distinct quiescence-inducing conditions, serum deprivation (SD), low-binding culture (LB) and contact inhibition (CI). QPCR-based analysis indicated increased Notch3 expression in response to each of these quiescence-inducing stimuli (**Fig. 2E**). Together, these results indicate that ectopic ΔNp63α and other quiescence-inducing stimuli enhance expression of Notch3. They also suggest that expression of Notch3 may mediate the effects of ΔNp63α and other quiescence-inducing stimuli.

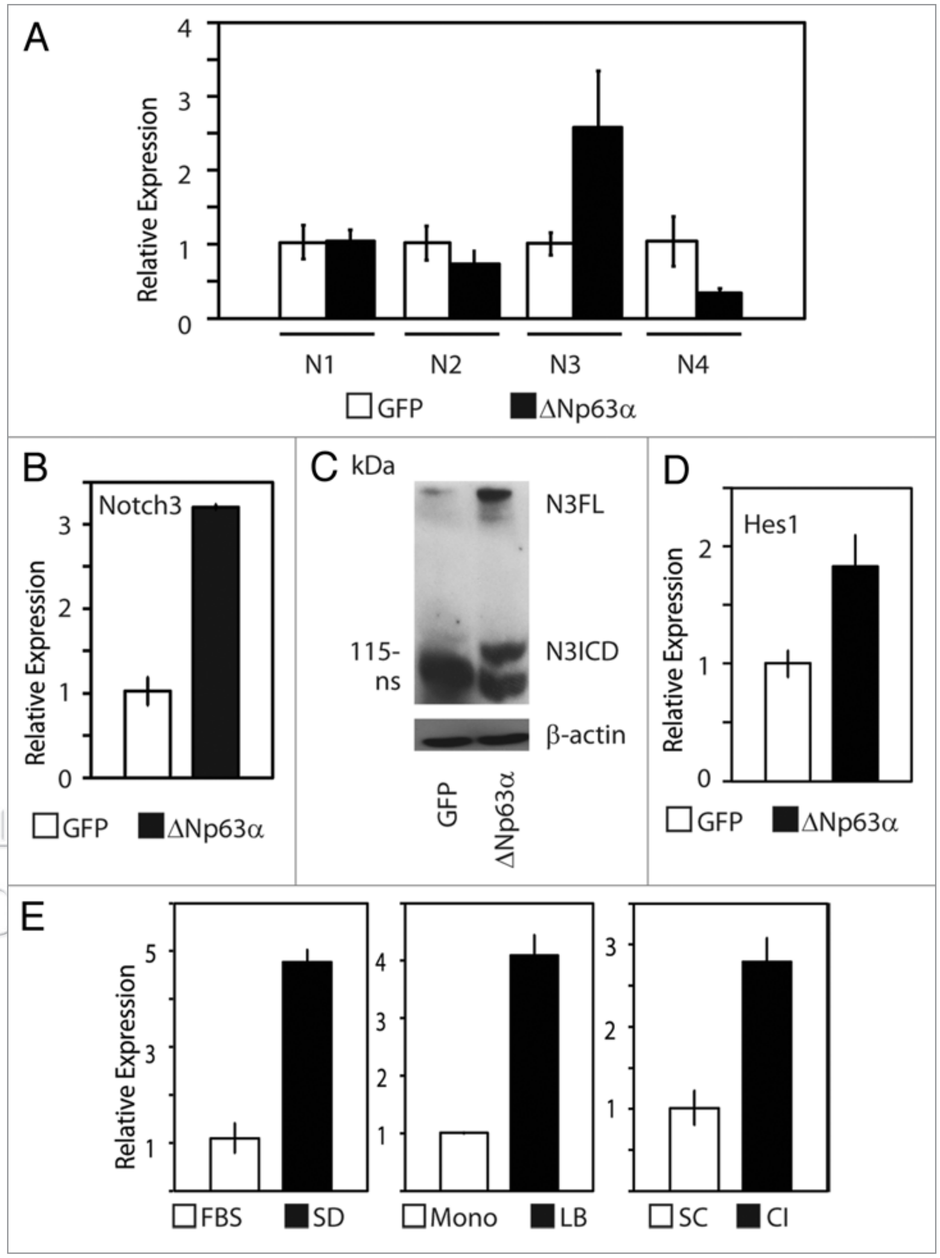

Figure 2. Notch3 is induced by ectopic ΔNp63α and other quiescence-inducing stimuli. (A) ΔNp63α specifically induces Notch3 mRNA in HC11 cells. Infection with adenovirus expressing ΔNp63α or GFP for 48 h significantly induced Notch3 mRNA as assessed by qPCR ($p < 0.05$, Student t-test). (B) ΔNp63α induces Notch3 mRNA in MCF7 breast cancer cells. (C) ΔNp63α activates signaling of the Notch3 receptor. In addition to elevating total Notch3, ΔNp63α overexpression also increases the amount of cleaved Notch3 intracellular domain, an indicator of active Notch signaling, as assessed by protein gel blot. (D) ΔNp63α induces Hes1, a canonical Notch signaling target and mediator of cellular quiescence. (E) Notch3 is induced following multiple quiescence-promoting stimuli in addition to ΔNp63α. As assessed by qPCR, Notch3 mRNA is elevated following serum deprivation (SD), low-binding culture (LB) and contact inhibition (CI) ($p < 0.05$).

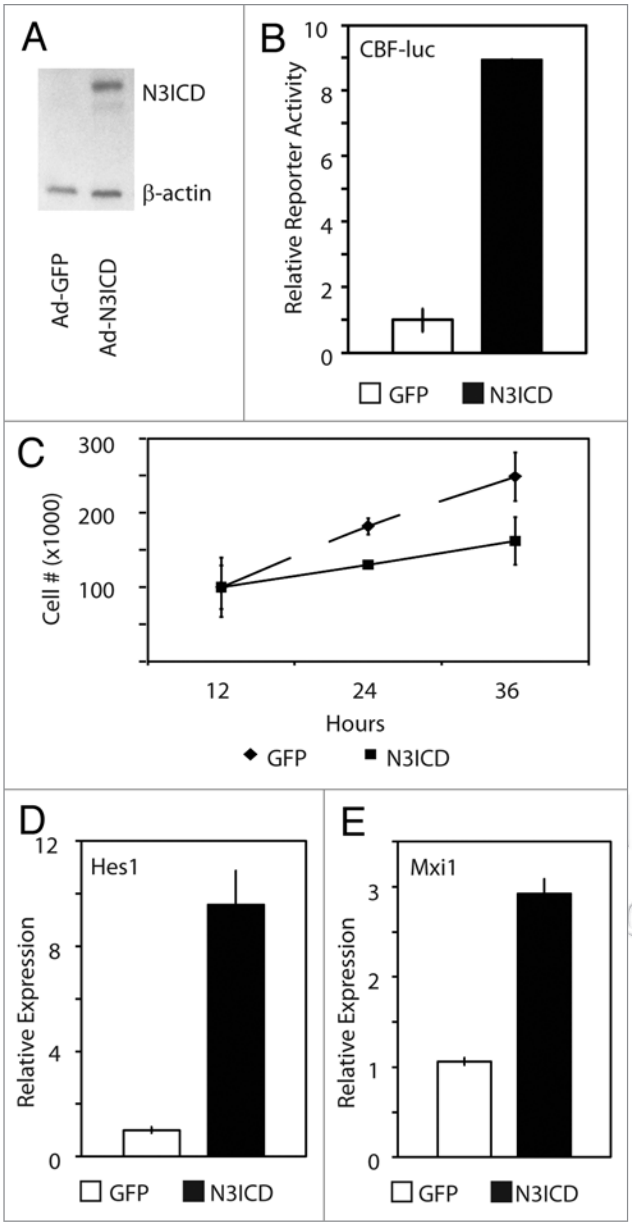

Figure 3. Constituative Notch3 activity is anti-proliferative and induces expression of quiescence-associated genes. (A) Overexpression of Notch3 intracellular domain ($N3^{ICD}$) by adenoviral infection of HC11 cells. β-actin as loading control. (B) Overexpression of $N3^{ICD}$ activates Notch signaling. Relative luciferase activity from CBF-luc reporter following GFP or $N3^{ICD}$ overexpression. (C) $N3^{ICD}$ suppresses HC11 proliferation similar to ΔNp63α. HC11 cells were trypsinized and counted every 12 h following plating and infection with adenoviral $N3^{ICD}$ or GFP. (D and E) $N3^{ICD}$ induces mRNA of canonical Notch signaling targets and quiescence-mediating genes. Overexpression of $N3^{ICD}$ increases mRNA of the canonical Notch signaling target and regulator of quiescence, Hes1 (C) and the c-myc-regulating mediator of quiescence, Mxi1 (D) as assessed by qPCR ($p < 0.05$).

Constituative Notch3 activity promotes quiescence. The previous data coupled to studies implicating Notch signaling in the governance of MaSCs proliferation[26] predict that Notch3 expression and signaling mediates ΔNp63α-induced quiescence, and that activation of Notch signaling in HC11 cells will promote quiescence. To test this, gain-of-function studies were performed by infecting HC11 cells with an adenovirus programmed to express the intracellular domain of Notch3 ($N3^{ICD}$) or GFP. Overexpression of $N3^{ICD}$ (**Fig. 3A**) but not GFP was sufficient to activate a Notch signaling reporter consisting of four CBF1-binding elements fused to the SV40 minimal promoter.[44] In similar studies, $N3^{ICD}$ had a potent anti-proliferative effect on HC11 cells (**Fig. 3C**) and was sufficient to induce Hes1 mRNA levels approximately 10-fold (**Fig. 3C**). The latter result indicates that ectopic $N3^{ICD}$ was sufficient to activate expression of a canonical Notch signaling target gene. Additionally, Hes1 is required to preserve the reversibility associated with quiescence.[25] In addition to Hes1, $N3^{ICD}$ also induced mRNA levels of Mxi1, a negative regulator of c-myc activity recently identified as a serum deprivation early response gene (SDERG) and shown to be essential for quiescence.[24] These studies demonstrate that Notch signaling is sufficient to arrest the growth of HC11 cells in a manner that is consistent with cellular quiescence.

Suppression of Notch3 disrupts quiescence and promotes expansion of self-renewing populations. The previous data indicated that ectopic Notch signaling reduced proliferation and increased expression of genes associated with cellular quiescence. This predicts that disruption of Notch signaling in HC11 cells might subvert quiescence. To test this, we sought to measure the effects of Notch disruption on BrdU incorporation rates following quiescence-inducing stimuli. Pharmacologic inhibition of Notch signaling with the γ-secretase inhibitor DAPT doubled the rate of BrdU incorporation following growth factor reduction (**Fig. 4A**), which supports the assertion that Notch-mediated growth arrest is reversible and consistent with cellular quiescence. Similarly, shRNA-mediated suppression of Notch3 (**Fig. 4E**) resulted in increased proliferation under growth factor-reduced conditions (**Fig. 4B**). These results indicate that disruption of Notch signaling or Notch3 expression is sufficient to confer resistance to quiescence-inducing stimuli and are consistent with previous studies indicating that suppression of Notch signaling in MaSCs results in mitotic expansion.[26] They also predict that suppression of Notch signaling will promote expansion of self-renewing subpopulations within HC11 cells. To test this, the mammosphere-forming capacity of HC11 cells was measured in the presence of DAPT or a vehicle control. Quantification of mammospheres indicated that inhibition of Notch signaling with DAPT caused a statistically significant increase in mammosphere initiation (**Fig. 4C**). Additionally, shRNA-mediated suppression of Notch3 resulted in greater mammosphere initiation relative to scrambled shRNA controls (**Fig. 4D**). These results demonstrate that Notch signaling exerts an anti-proliferative effect on self-renewing populations within the HC11 cell culture system.

Disruption of Notch signaling blocks ΔNp63α-mediated cellular quiescence. Data presented here support a model in which ΔNp63α promotes quiescence by increasing Notch3 expression and activity. This model predicts that suppression of Notch3 expression will disrupt ΔNp63α-mediated cellular quiescence. We sought to compare the effects of ectopic ΔNp63α on proliferation by HC11 derivatives programmed to express a scrambled shRNA or a Notch3-directed shRNA. Results (**Fig. 5A**) indicate that suppression of Notch3 subverted the anti-proliferative effects of ΔNp63α. Additionally, suppression of Notch3 expression disrupted ΔNp63α-mediated accumulation of cells in the 2N state (**Fig. 5B**). Finally, suppression of Notch3 significantly reduced ΔNp63α-mediated accumulation of pyronin Y^{low}-staining cells. Together, these results demonstrate that suppression of Notch3 expression (**Fig. 5C**) is sufficient to subvert ΔNp63α-mediated cellular quiescence. These data strongly support a model in which the ability of ΔNp63α to increase expression and activity of Notch3 is functionally linked to the ability of ΔNp63α to promote cellular quiescence.

Discussion

Studies indicate that label-retaining cells co-purify with tissue-specific adult stem cells, suggesting a physiologic role for quiescence in preservation of self-renewal within regenerative hierarchies that govern development, stasis, aging and cancer. Central to this model is the functional asymmetry of stem cell division that yields mitotic siblings with distinct fates. While one sibling forfeits self-renewing capacity and enters a stage of transient amplification, a second retains it and enters a state of quiescence. Doing so enables retention of proliferative capacity and evasion of the negative effects of excessive cell division, including telomeric erosion, accumulation of reactive oxygen species and increased risk of mutation. Quiescence is also a potent blockade to differentiation,[11] suggesting a role in developmental potency. Despite the critical role of quiescence, the molecular and genomic events associated with entry into and maintenance of quiescence are incompletely understood. Work presented here describes a regulatory relationship between ΔNp63α and Notch3 that governs quiescence and demonstrates for the first time that ΔNp63α promotes quiescence and suggests a mechanism by which ΔNp63α promotes stem cell longevity.

While the hypotheses surrounding the role of adult stem cells in cancer initiation and etiology remain controversial and unproven, there is abundant evidence indicating that diverse tumors possess a subpopulation of cells that are uniquely tumorigenic and able to self-renew. Multiple studies have demonstrated that this subpopulation displays broad-spectrum resistance to cytotoxic chemotherapeutics and ionizing radiation,[45-47] thereby implicating this subpopulation in cancer recurrence. Other studies demonstrate a correlation between label retention and chemoresistance in cancer models, suggesting that cellular quiescence may confer resistance to therapeutics that target proliferating cells.[23] Consistent with this are studies indicating that subversion of quiescence in leukemic stem cells renders these cells sensitive to chemotherapeutics.[48] Therefore, targeting genetic pathways governing stem cell quiescence in the setting of adjuvant therapeutics represents a promising strategy to reduce cancer recurrence. Here, we present data indicating that disruption of Notch signaling subverts quiescence in a cell culture model with features of MaSCs. Additionally, we demonstrate that Notch signaling may mediate a cellular response to diverse quiescence-inducing stimuli, including ΔNp63α activity, suggesting a fundamental role in cellular quiescence that may apply to multiple cancer stem cell models.

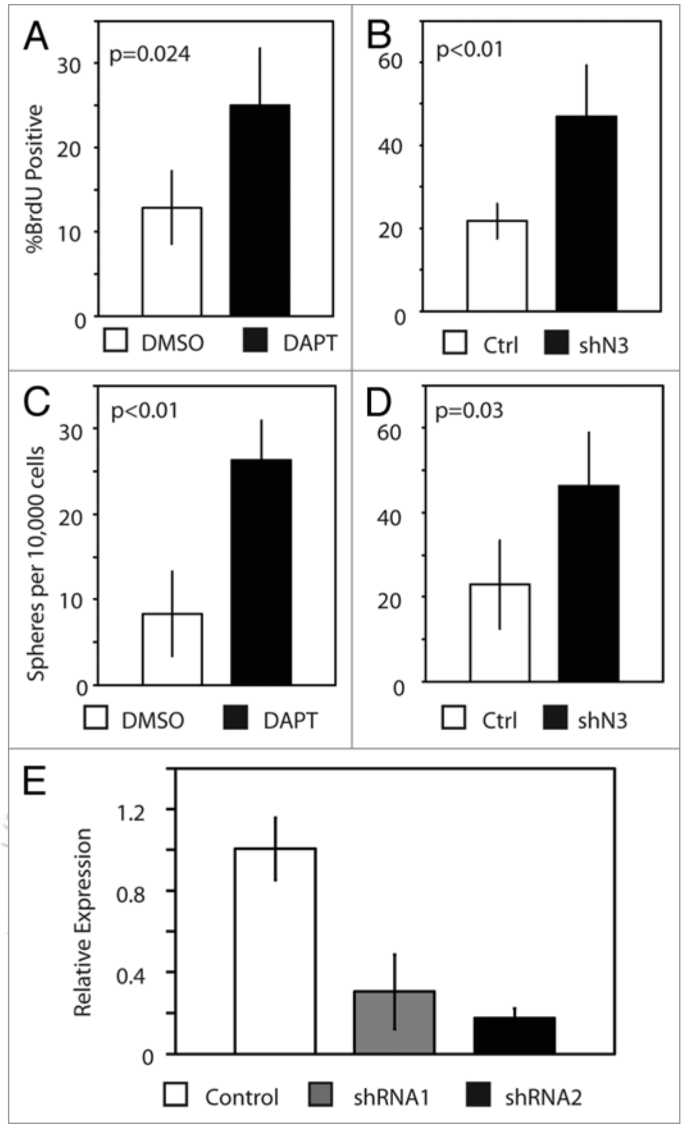

Figure 4. Disruption of Notch3 expression or activity prevents quiescence and promotes the expansion of self-renewing populations. (A) Pharmacologic inhibition of Notch signaling suppresses quiescence. Following serum deprivation in the presence of the γ-secretase inhibitor DAPT or control, DMSO, HC11 cells were pulsed with BrdU and fixed. Immunostaining for BrdU incorporation followed by counting of BrdU+ cells showed an increase in the number of cells synthesizing DNA in the presence of DAPT despite the quiescence-inducing effects of serum deprivation. (B) Genetic inhibition of Notch3 suppresses quiescence. Cells assayed as in (A), in the presence of Notch3-specific shRNA or control: a similar trend was seen when specifically targeting the Notch3 receptor. (C) DAPT treatment suppresses attachment-independent induced quiescence. Inhibiting Notch signaling increased the number of spheres formed in low-binding culture. (D) Notch3 suppression by shRNA also blocks attachment-independent induced quiescence. (E) Suppression of Notch3 mRNA by two independent Notch3-targeting shRNAs assessed by qPCR.

Cellular and developmental context are critical determinants of Notch signaling output that may account for the diverse cellular responses to perturbations in Notch signaling.[27-30] This diversity is best illustrated by abundant and compelling evidence that

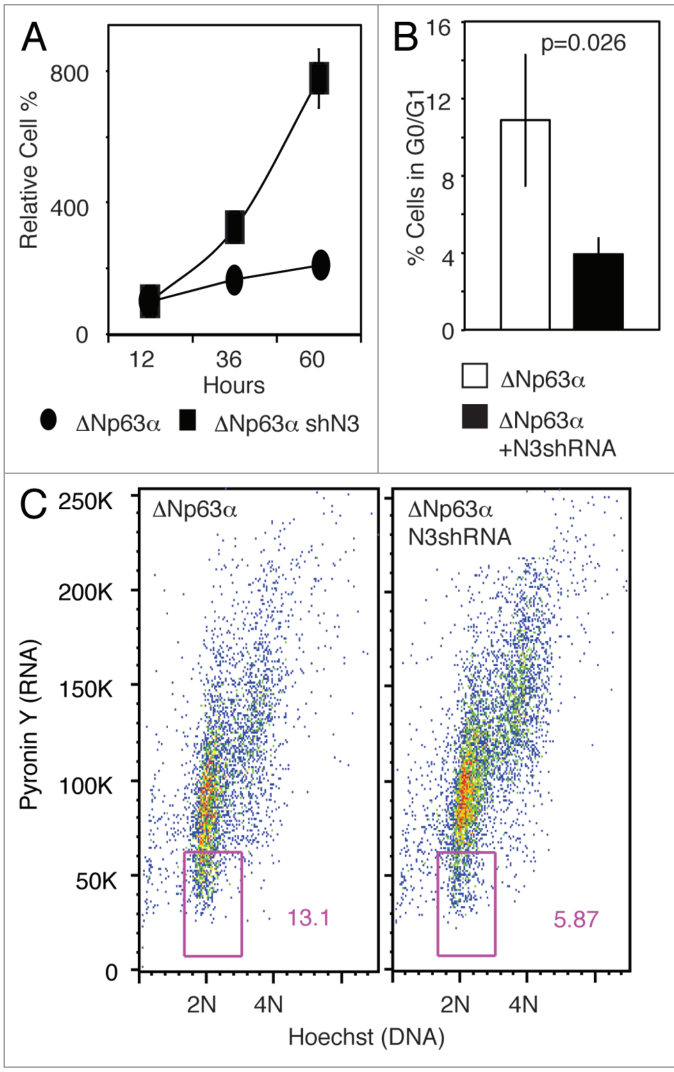

Figure 5. (A) Notch3 shRNA abrogates ΔNp63α suppression of proliferation. HC11 cells expressing Notch3 shRNA or control were counted every 12 h following adenoviral expression of ΔNp63α. (B) Notch3 suppression blocks the G_0/G_1 arrest induced by ΔNp63α. HC11 cells expressing Notch3 shRNA or control were treated as in **Figure 1C**. Bar graph depicts the average number of cells with 2N DNA content. (C) Suppression of Notch3 blocks the quiescence inducing effects of ΔNp63α. HC11 cells expressing Notch3 shRNA or control were infected with ΔNp63α-expressing adenovirus and assessed as in **Figure 1D**.

Notch signaling can be either oncogenic or tumor suppressive in distinct cellular contexts. A model that may account for this variability holds that Notch signaling instructs mutually exclusive cell fates upon Notch donor and recipient cells via lateral inhibition;[42] however, the molecular determinants of context specificity are largely unidentified. Within the context of governance of stem cell quiescence, our study suggests that ΔNp63α may be one such determinant of Notch signaling output. Additionally, it is unclear whether the four Notch family members mediate identical Notch signaling outputs. In this manuscript, we reported that while only Notch3 was responsive to ΔNp63α, both Notch1 and Notch2 were expressed at higher levels. This may suggest that effects of DAPT are due to inhibition of signaling from multiple Notch receptors. In this manuscript we present data indicating that shRNA-mediated ablation of Notch3 was sufficient to prevent quiescence following growth factor reduction. Our data indicate that Notch signaling is anti-proliferative and promotes expression of genes associated with quiescence and support a model in which activation of Notch by ΔNp63α represents a mechanism by which stem cell quiescence is maintained. Consistent with this model is the remarkable finding that the mammary glands of MMTV-Notch1^{ICD} and MMTV-Notch3^{ICD} undergo a developmental blockade that disrupts lobulo-alveolar development in pregnant mice.[36] This is in contrast to several other MMTV-based breast cancer models characterized by precocious lobulo-alveolar development and is consistent with a model in which Notch signaling suppresses activation of the mammary regenerative hierarchy.

Two recent studies have also implicated p53 in the governance of stem cell activity. One identified necdin as a gene that is regulated by p53 in the absence of any cellular or genotoxic stress and showed that necdin was necessary to maintain the ratio of long-term hematopoetic stem cells to short-term hematopoetic stem cells.[49] A second study showed that p53$^{-/-}$ mammary epithelial cells had greater mammosphere-forming capacity and greater mammary regenerative capacity than mammary epithelial cells from wild-type counterparts.[50] Importantly this study showed that the increased regenerative activity that resulted from p53 ablation was neutralized by DAPT, suggesting strongly that Notch signaling was activated in response to p53 suppression. This study also showed that long-term BrdU retention was compromised in p53$^{-/-}$ mice. Collectively, these studies indicate that a complex relationship between ΔNp63 isoforms and p53 may underlie the governance of quiescence vs. activation in adult stem cell populations.

Our conclusion that ΔNp63α promotes cellular quiescence coupled to studies indicating that it is required to avoid cellular senescence[40,51,52] suggests a dynamic model in which ΔNp63α balances quiescence and senescence to preserve long-term replicative capacity and a prolonged life span. While there is abundant evidence that p53 is a potent inducer of cellular senescence, more recent studies have indicated that it can also promote quiescence and, in so doing, prevent senescence. Importantly, this study demonstrated that the ability of p53 to induce quiescence is the result of p53-mediated suppression of senescence.[53] While the cellular and molecular mechanisms underlying this paradox are incompletely understood, another recent study has implicated the status of the mTOR signaling pathway in the p53-mediated outcome.[54] Additionally, ΔNp63α has been shown to be a transcriptional target of p53.[55] This coupled to the fact that ΔNp63α is expressed in a highly cell-type dependent manner suggests a model in which cells that are capable of p53-dependent regulation of ΔNp63α may be prone to quiescence, while those in which p53 is present but ΔNp63α expression is repressed may be prone to senescence.

Materials and Methods

Cell culture. HC11 cells (a kind gift from Sergei Tevosian) were cultured in RPMI-1640 with L-glutamine (Mediatech Inc.)

supplemented with 10% FBS, 5 ug/ml Insulin, 10 ng/ml EGF, 100 units/ml Penicillin, 100 ug/ml Streptomycin, and MCF7 cells were cultured in DMEM supplemented with 10% FBS at 37°C and 5% CO_2. Serum deprivation studies involved culturing cells in a 100-fold dilution of normal media for 14 h. Low-binding assays were performed in 24-well Ultra Low Cluster plates (Corning) with normal media. Sphere counting was done using ImageJ software based on three low power images for each sample. Following growth to confluence, media was replaced and cells were maintained for two additional days to allow for contact inhibition. Cell counting experiments were done following trypsinization and counting by hemocytometer.

Cell cycle analysis. For cell cycle analysis based on propidium iodide or Hoechst/pyronin staining followed by flow cytometry, cells were treated as described, detached by trypsinization, resuspended in PBS, fixed in a final concentration of ice-cold 70% EtOH and stored overnight at -20°C prior to analysis. For propidium iodide staining, cells were washed in PBS, resuspended in PBS with 0.1% Triton X-100, 0.2 mg/ml DNase-free RNase A and 40 ul of 500 ug/ml propidium iodide stock and incubated at 37°C for 30 min. For Hoechst/pyronin staining, cells were washed in HBSS with Ca^{2+} and Mg^{++} and resuspended in HBSS containing 1.2 ug/ml Hoechst 33,342 and 2 ug/ml pyronin Y. Flow cytometry was performed on a BD FacScan or BD FacsAria for propidium iodide or Hoechst/pyronin Y, respectively. For synchronization, cells were treated with 2 mM thymidine or 100 ng/ml nocodazole. To assess S-phase fraction, cells were pulsed with 10 uM Bromodeoxy Uridine (BrdU) for 20 min followed by fixation with CytoRich Red (Thermo Fisher Scientific). BrdU was detected with mouse anti-BrdU (BD Bioscience) and goat anti-mouse AlexaFluor 568 (Invitrogen). Number of BrdU-positive cells per total cells from four high power fields were counted for each sample.

Adenovirus and lentivirus. ΔNp63α and Notch3 intracellular domain were sub-cloned from pcDNA3.1 into pShuttleCMV and further recombined through the AdEasy Adenoviral production system using HEK-293Ad cells. Adenovirus was used as 1,000x for all reactions. pLKO.1-Notch3 shRNAs (OpenBiosystems, TRCN0000075571 "shRNA1," TRCN0000075572 "shRNA2") were cotransfected with packaging and envelope containing plasmids into HEK-293T cells. Virus-containing media was used to infect HC11 cells, and stable expressing populations were selected and grown in the presence of puromycin. Data from shRNA1 expressing cells are shown unless otherwise specified.

Notch inhibition. Cleavage of Notch receptors was blocked by inhibition of γ-secretase with 10 μM DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, Sigma).

Luciferase reporter assay. DNA fragment coding for EGFP was removed from CBFRE-EGFP (Addgene) and replaced with DNA fragment coding for luciferase to produce CBF-luc plasmid. Cells were treated as described and transfected with CBF-luc and renilla for 24 h. Luciferase activity was assessed with Dual-Luciferase Reporter Assay System (Promega).

Quantitative PCR and protein gel blotting. RNA was collected with the RNEasy kit (QIAGEN), and cDNA was prepared using the iScript cDNA Synthesis kit (BioRad). QPCR was performed with oligonucleotide primers specific for each target with iQ SYBR Green Super mix (BioRad) and analyzed with Bio-Rad CFX Manager software. Normalization with GAPDH was done using the $2^{-\Delta\Delta Ct}$ method. Protein gel blotting of protein lysates prepared with NET-N lysis buffer following PAGE was performed with antibodies detecting PARP (rabbit, Cell Signaling Inc.-), p63 (mouse, 4A4, Abcam), Notch3 (goat, M-20, Santa Cruz) or β-actin (mouse, 8H10D10, Cell Signaling Inc.).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by grants from the National Cancer Institute (RO1CA108539) to J.D.R. and the Elsa U. Pardee Foundation to J.D.R. S.K. was supported by a fellowship from the Rosaline Borison Memorial Predoctoral Fellowship.

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